THE CD40CR RECEPTOR AND LIGANDS THEREFOR

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THE CD40CR RECEPTOR AND LIGANDS THEREFOR

1. INTRODUCTION

The present invention relates to a counter-
5 receptor, termed CD40CR, for the CD40 B-cell antigen,
and to soluble ligands for this receptor, including
fusion molecules comprising at least a portion of CD40
protein. It is based, at least in part, on the
discovery that a soluble CD40/immunoglobulin fusion
10 protein was able to inhibit helper T-cell mediated B-
cell activation by binding to a novel 39 kD protein
receptor on helper T-cell membranes. The present
invention provides for a substantially purified CD40CR
receptor; for soluble ligands of CD40CR, including
15 antibodies as well as fusion molecules comprising at
least a portion of CD40 protein; and for methods of
controlling B-cell activation which may be especially
useful in the treatment of allergy or autoimmune
disease.
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2. BACKGROUND OF THE INVENTION

Studies by Mitchison, Benacerraf and Raff first
suggested that physical interactions between T_h and B-
cells were essential in the development of humoral
25 immune responses. Later studies documented that T_h
formed physical conjugates with class II major
histocompatibility complex (MHC) compatible, antigen-
presenting B-cells (Vitetta et al., (1987) Immunol.
Rev. 99:193-239) and that it was the B-cells within
30 these conjugates that responded to T_h (Bartlett et al.,
(1989) J. Immunol. 143:1745-1754). With the discovery
that T_h-derived lymphokines exerted potent growth and
differentiative effects on B-cells, it was proposed
that soluble factor(s) released in proximity by
35 activated T_h mediated the activation of the interacting

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B-cell. However, none of the molecularly cloned lymphokines, alone or in combination, manifested the ability to induce B-cell cycle entry. Unlike soluble factors, plasma membrane fractions from activated T_h

5 induced B-cell cycle entry (Hodgkin et al., (1990) J. Immunol. 145:2025-2034; Noelle et al., (1991) J. Immunol. 146:1118-1124). Studies using purified plasma membrane fractions from activated T_h suggested that a protein expressed on the membrane of activated

10 T_h was responsible for initiating humoral immunity (Noelle et al., (1991) J. Immunol. 146:1118-1124; Bartlett et al., (1990) J. Immunol. 145:3956-3962).

Purified plasma membranes from activated T_h (PM^Ac) have been used to investigate the nature of this

15 effector function (Hodgkin et al. (1990) J. Immunol. 145:2025-2034; Noelle et al., (1991) J. Immunol. 146:1118-1124). PM^Ac from activated T_h, but not resting T_h (PM^rd) expressed an activity that induced B-cell cycle entry in an antigen-nonspecific, class II-

20 unrestricted manner. In addition, it was shown that the activity expressed by PM^Ac required 4-6 hours of activation, de novo RNA synthesis and was protein in nature (Bartlett et al., (1990) J. Immunol. 145:3956-3962).

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SUMMARY OF THE INVENTION

The present invention relates to a counter-receptor, termed CD40CR, for the CD40 B-cell antigen, and to soluble ligands for this receptor, including

30 fusion molecules comprising at least a portion of CD40 protein. It is based, at least in part, on the discovery that a soluble CD40/immunoglobulin fusion protein was able to inhibit helper T-cell mediated B-cell activation by binding to a novel 39 kD receptor

35 protein (termed "CD40CR" for CD40 counter-receptor) on

helper T-cell membranes, and on the discovery that a monoclonal antibody, termed MR1, directed toward this 39 kD receptor was able to inhibit helper T-cell mediated activation of B-cells.

5   The present invention provides for a substantially purified CD40CR receptor; for soluble ligands of CD40CR, including antibodies as well as fusion molecules comprising at least a portion of CD40 protein; and for methods of controlling B-cell

10   activation.

In particular embodiments of the invention, B-cell activation in a subject may be inhibited by contacting helper T cells of the subject with effective amounts of a soluble ligand of CD40CR. Such

15   inhibition of B-cell activation may be especially useful in the treatment of allergy or autoimmune disease.

One advantage of the present invention is that it enables intervention in an aspect of the immune

20   response which is not antigen specific. Many current therapies for allergy include desensitization to particular antigens, and require that each patient be tested in order to identify antigens associated with sensitivity. As a practical matter, exhaustive

25   analysis of a patient's response to each and every potential allergen is virtually impossible. Furthermore, in most autoimmune conditions, the causative antigen is, generally, unknown or even irrelevant to the disease process. The present

30   invention, which relates to the antigen nonspecific CD40/CD40CR interaction, circumvents the need to characterize the antigen associated with allergy or autoimmunity. Therefore, the present invention may be used to particular advantage in the treatment of

35   allergic conditions in which the immunogen is not

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known, or has multiple components, for example, in hay fever or in procainamide induced lupus. It may also be useful in acute treatment of immune activation, for example, in therapy for anaphylaxis.

3.1. ABBREVIATIONS

Ig	immunoglobulin
mab	monoclonal antibody
PM^{Act}	plasma membranes prepared from activated helper T-cells
PM^{rest}	plasma membranes prepared from resting helper T-cells
PAGE	polyacrylamide gel electrophoresis
rIL4	recombinant interleukin 4
rIL5	recombinant interleukin 5
SN	supernatant
T_h	helper T-cell
T_h1	refers to D 1.6, a I-A^d-restricted, rabbit immunoglobulin specific clone

4. DESCRIPTION OF THE FIGURES

Figure 1. Effect of monoclonal antibodies and CD40-Ig on the induction of B-cell RNA synthesis by PM^{Act}.

Panel A. Resting B-cells were cultured with PM^{rest} or PM^{Act} from T_h1. 25µg/ml of anti-CD4, anti-LFA-1 or anti-ICAM-1 or a combination of each of these (each at 25 µg/ml) was added to wells containing PM^{Act} and B-cell RNA synthesis was measured by incorporation of [^{3}H]-uridine. B-cell RNA synthesis was assessed from 42 to 48 hours post-culture. Results presented are the arithmetic means of triplicate cultures +/- s.d., and are representative of 5 such experiments.

Panel B. Resting B-cells were cultured with PM^{Act} from T_h1 (●, ▲) or T_h2 (□). To the T_h1 PM^{Act}

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containing cultures (●, ▲), increasing amounts of
CD40-Ig (▲) or control protein CD7E-Ig(♦) were added.
To the T_h2 PM^{Act} containing culture (□), increasing

5   amounts of CD40-Ig were added. B-cell RNA synthesis
was assessed from 42 to 48 hours post-culture.
Results presented are the arithmetic means of
triplicate cultures +/- s.d., and are representative
of 3 such experiments.

10   Panel C. Resting B-cells were cultured with
LPS (50 µg/ml) or PM^{Act}. To cultures, CD40-Ig (25
µg/ml; hatched) or CD7E-Ig (25 µg/ml; solid) were
added. RNA synthesis was determined as described in
Panel A. Results presented are the arithmetic mean of

15 triplicate cultures +/- s.d., and are representative
of 3 such experiments.

        Figure 2. CD40-Ig inhibited B-cell
differentiation and proliferation.

20   Panel A. Resting B-cells were cultured with
PM^{Act}, rIL4 (10 ng/ml) and rIL5 (5 ng/ml). Either at
the initiation of culture, or on days 1, 2 or 3 post-
initiation of culture, CD40-Ig or CD7E-Ig (25 µg/ml)
were added. On day six of culture, SN from individual

25 wells were harvested and quantitated for IgM(■) and
IgG$_1$(●) using an anti-isotype specific ELISA, as
described in (Noelle et al., (1991) J. Immunol.
$\underline{146}$:1118-1124). In the presence of PM^{Act}, IL4 and IL5,
(in the absence of added CD40-Ig) the concentrations

30 of IgM and IgG$_1$ were 4.6 µg/ml and 126 ng/ml,
respectively. Cultures which received CD7E-Ig (25
µg/ml) on Day 0 produced 2.4 µg/ml and 89 ng/ml of IgM
and IgG$_1$, respectively. In the absence of IL4 and IL5,
no IgM or IgG$_1$ was detected. Results are representative

35 of 3 such experiments.

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Panel B. T_h1 were rested or activated with anti-CD3 for 16 hours, irradiated and cultured (1×10^4/well) with resting B-cells (4×10^4/culture) in the presence of IL4 (10 ng/ml). Between 0 and 25

5 μg/ml of CD40-Ig (▲) or CD7E-Ig (●) were added to cultures. From 66-72 hours post-culture, wells were pulsed with 1.0 μCi of [^{3}H]-thymidine and harvested. The dotted line indicates the response of B-cells to resting T_h. Results presented are the arithmetic mean

10 of triplicate cultures +/- s.d., and are representative of 2 such experiments.

Figure 3. CD40-Ig detected a molecule expressed on activated, but not resting T_h. Resting and

15 activated T_h were harvested and incubated with fusion proteins for 20 minutes at 4°C, followed by FITC-conjugated goat anti-hIgG (25 μg/ml). Percentage positive cells and MFI were determined by analysis of

20 at least 5000 cells/sample. Results are representative of 6 such experiments. CD40-Ig binding is indicated by a filled-in profile.

Figure 4. CD40-Ig immunoprecipitated a 39 kD

25 protein from lysate of activated T_h1. T_h1 were rested or activated with insolubilized anti-CD3 for 16 hours. [^{35}S]-labelled proteins from resting or activated T_h were immunoprecipitated with purified antibodies or fusion proteins (1-10μ). The gel profile is

30 representative of 3 such experiments.

Figure 5. A monoclonal antibody (mAb), specific to the induced 39 Kd T_h membrane protein, inhibited induction of B-cell RNA synthesis by PM^{Ad}. Resting B-

35 cells and PM^{Ad} were cultured with 10 μg/ml each of anti-α/β, anti-CD3, CD40-Ig or MR1. RNA synthesis was

determined as described in Figure 1. Results presented are the arithmetic means of triplicate cultures +/- s.d., and are representative of 3 such experiments.

Figure 6. MR1 and CD40-Ig recognized the same molecule expressed on activated T_h.

Panel A: Activated T_h were fluorescently stained with MR1 or control Ig. To evaluate if CD40-Ig and MR1 competed for binding to activated T_h, graded concentrations of MR1 or control hamster Ig (anti-α/β TCR) were added together with anti-CD40 (20 μg/ml). After incubation for 20 minutes at 4°C, the samples were washed and incubated with FITC-conjugated, mab anti-human IgG_1. Results are representative of 3 such experiments.

Panel B: Proteins from [^{35}S]-methionine-labelled, activated T_h were immunoprecipitated with MR1 (10 μg/sample) or CD40-Ig (10 μg/sample) and resolved by PAGE and fluorography. Results presented are representative of 2 such experiments.

Figure 7. Binding of CD40-Ig to human cell lines. A variety of human T-cell lines were exposed to biotin-labelled CD40-Ig, and binding was evaluated by flow cytometry.

Figure 8.

Panel A: Nucleotide sequence of CD40 cDNA from Stamenkovic et al., (1989) EMBO J. 8:1403-1410. The transmembrane region is underscored.

Panel B: Schematic diagram of a plasmid that may be used to express CD40-Ig. The amino acid sequences at the site of fusion of Δ CD40 is shown below the diagrammed portion of CD40.

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5. DETAILED DESCRIPTION OF THE INVENTION

The present invention provides for Δsubstantially purified CD40CR receptor; for soluble ligands of CD40CR, including antibodies as well as fusion

5   molecules comprising CD40; and for methods of controlling B-cell activation.

For purposes of clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the following subsections:

10   (i)   ligands that bind to CD40CR;

            (ii)   methods used to characterize CD40CR;

            (iii)   preparation of purified CD40CR;

            (iv)   uses of ligands that bind to CD40CR; and

15   (v)   uses of CD40CR.

5.1. LIGANDS THAT BIND TO CD40CR

The present invention provides for soluble ligands of CD40CR, including (i) fusion molecules

20   comprising at least a portion of CD40 protein and (ii) antibodies or antibody fragments.

The term "soluble," as used herein, indicates that the ligands of the invention are not permanently associated with a cell plasma membrane. Soluble

25   ligands of the invention may, however, be affixed to a non-cellular solid support, including a lipid, protein, or carbohydrate molecule, a bead, a vesicle, a magnetic particle, a fiber, etc. or may be enclosed within an implant or vesicle.

30   The ability of such a ligand to bind to CD40CR may be confirmed by demonstrating that the ligand binds to the same protein as CD40-Ig (infra) or MR1 (infra).

35

The ligands of the invention may be comprised in pharmaceutical compositions together with a suitable carrier.

5.1.1.   FUSION MOLECULES

The present invention provides for soluble fusion molecules that are ligands of CD40CR. Such fusion molecules comprise at least a portion of CD40 protein attached to a second molecule. The portion of CD40 preferably lacks the CD40 transmembrane domain. A portion of CD40 protein which may be used according to the invention is defined as any portion which is able to bind to CD40CR, for example, such a portion may be shown to bind to the same protein as MRl or CD40-Ig.

Second molecules which may be used include peptides and proteins, lipids, and carbohydrates, and, in preferred embodiments of the invention, may be an immunoglobulin molecule, or portion thereof (such as an Fv, Fab, F(ab')$_2$, or Fab' fragment) or CD8, or another adhesion molecule, such as B7. The second molecule may be derived from either a non-human or a human source, or may be chimeric. The second molecule may also be an enzyme, toxin, growth factor, lymphokine, antiproliferative agent, alkylating agent, antimetabolite, antibiotic, vinca alkaloid, platinum coordinated complex, radioisotope, or a fluorescent compound.

The fusion molecules of the invention may be produced by chemical synthesis or, preferably, by recombinant DNA techniques.

For example, a nucleic acid sequence encoding at least a portion of CD40 protein may be combined with a nucleic acid sequence encoding a second molecule in a suitable expression vector, and then expressed in a prokaryotic or, preferably, eukaryotic expression

system, such as a yeast, baculovirus, or mammalian expression system, including transgenic animals.

Alternatively, at least a portion of CD40 protein may be expressed using recombinant DNA techniques and then may be chemically conjugated to a second molecule.

Fusion molecules comprising CD40 may be purified from preparative mixtures using electrophoretic techniques or affinity chromatography using ligand that binds to either CD40 or to the second molecule. Ligands that bind to CD40 include, but are not limited to, anti-CD40 antibodies such as G28-5, as produced by the hybridoma having accession number HB9110 and deposited with the American Type Culture Collection, and CD40CR, described more fully in sections 5.2 and 5.3, infra. If the second molecule is an immunoglobulin or immunoglobulin fragment, an affinity column comprising anti-immunoglobulin antibody may be used; if the second molecule comprises an F_c fragment, a protein A column may be used.

According to a preferred embodiment of the invention, a portion of CD40 may be produced using a nucleic acid sequence that encodes a CD40 protein that is truncated upstream from the transmembrane domain. Such a nucleic acid sequence may be prepared by digesting a plasmid containing a cDNA encoding CD40 antigen, such as that described in Stamenkovic et al., (1989), EMBO J. 8:1403-1410, with PstI (P) and Sau 3A (S3) restriction enzymes. The resulting P/S3 fragment may be subcloned into the same plasmid digested with P and Bam HI (B), to produce a truncated CD40 gene (see Figure 8).

In particular, nonlimiting, embodiments of the invention, an expression vector used to produce ligands containing at least a portion of CD40 as well

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as immunoglobulin sequence may preferably comprise a virally-derived origin of replication, a bacterial origin of replication, a bacterial selectable marker, and eukaryotic promoter and enhancer sequences

5 separated from DNA sequences encoding an immunoglobulin constant region by restriction endonuclease sites which allow subcloning of DNA sequenes encoding at least a portion of CD40, followed by a polyadenylation signal sequence (see Figure

10 8.b.).

In a specific embodiment of the invention, the truncated CD40 gene may be subcloned into an immunoglobulin fusion plasmid, such as that described in *Aruffo et al., 1990, Cell 61:1303-1313*, using an

15 *Mlu I* and B digest, to form plasmid pCD40-Ig, which encodes the fusion molecule CD40-Ig (see Figure 8). CD40-Ig fusion protein may then be produced by transfecting the pCD40-Ig plasmid into COS cells to form a transient expression system. CD40-Ig produced

20 may be collected from the COS cell supernatant and purified by protein A column chromatography as described in *Aruffo et al., 1990, Cell 161:1303-1313*.

5.1.2. ANTIBODIES

25 The soluble ligands of the invention may comprise antibody molecules, monoclonal antibody molecules, or fragments of these antibody molecules which contain an antigen combining site that binds to CD40CR. Such ligands may further comprise a second molecule which

30 may be a protein, lipid, carbohydrate, enzyme, toxin, growth factor, lymphokine, antiproliferative agent, alkylating agent, antimetabolite, antibiotic, vinca alkaloid, platinum coordinated complex, radioisotope, or a fluorescent compound and may be linked to the

35 antibody molecule or fragment.

Where the ligand is a monoclonal antibody, or a fragment thereof, the monoclonal antibody can be prepared against CD40CR using any technique which provides for the production of antibody molecules by continuous cell lines in culture. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497) as well as other techniques which have more recently become available, such as the human B-cell hybridoma technique (Kozbar et al., 1983, Immunology Today 4:72) and EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96) and the like are within the scope of the present invention.

Antibody fragments which contain the idiotype of the molecule could be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')$_2$ fragment which can be generated by treating the antibody molecule with pepsin; the Fab' fragments which can be generated by reducing the disulfide bridges of the F(ab')$_2$ fragment; the F(ab')$_2$ fragment which can be generated by treating the antibody molecule with papain; and the 2Fab or Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent to reduce the disulfide bridges.

The present invention also provides for chimeric antibodies produced by techniques known in the art, such as those set forth in Morrison et al., (1984) Proc. Natl. Acad. Sci. U.S.A. 81:6851-6855 or European Patent Application No. 85305604.2, publication No. 0173494 by Morrison et al., published March 5, 1986.

Immunogen for the production of antibodies may be any source that contains CD40CR. For example, activated T$_h$ may be used as an immunogen.

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Alternatively, substantially purified CD40CR, prepared as set forth in section 5.3, _infra_, may be used. If activated T_h are used as immunogen, antiserum may be tested for reactivity against activated but not resting T_h cells.

In a preferred embodiment of the invention, the soluble ligand is the MR1 monoclonal antibody. The following method was used to produce the MR1 monoclonal antibody, and may be used to generate other antibodies directed toward CD40CR.

Hamsters were immunized intraperitoneally with 5-10^6 activated T_h1 cells (D1.6) at weekly intervals for six weeks. When the serum titer against murine T_h1 was greater than about 1:10,000, cell fusions were performed with polyethylene glycol using immune hamster splenocytes and NSI. SN from wells containing growing hybridomas were screened by flow cytometry on resting and activated T_h1. One particular hybridoma, which produced a mab that selectively recognized activated T_h, was further tested and subcloned to derive MR1. MR1 was produced in ascites and purified by ion exchange HPLC.

The present invention also provides for ligands comprising monoclonal antibodies, and fragments thereof that are capable of competitively inhibiting the binding of MR1 to its target antigen or CD40-Ig to its receptor.

5.2. METHODS USED TO CHARACTERIZE CD40CR

CD40CR may be characterized by (i) its ability to bind CD40, fusion molecules comprising at least a portion of CD40, and antibodies such as MR1; (ii) its functional characteristic of being able to stimulate B-cell cycle entry, proliferation, and differentiation; and (iii) its cellular distribution.

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5.2.1. ABILITY TO BIND LIGANDS

CD40CR may be characterized by its ability to bind to ligands such as CD40, fusion molecules comprising CD40, and antibodies directed toward CD40CR.

As discussed in greater detail _infra_, several techniques were used to characterize CD40CR. For example, CD40-Ig and MR1 were shown to recognize the same 39 kD molecule. Both CD40-Ig and MR1 were found to immunoprecipitate a 39 kD protein from radiolabelled T_h lysates (Figure 5b). Further, immunoprecipitation of the 39 kD protein with CD40-Ig removed the antigen recognized by MR1 from T_h lysates.

5.2.2. ABILITY TO STIMULATE B-CELLS

CD40CR may also be characterized by its ability to stimulate B-cell cycle entry, proliferation, and differentiation.

For example, plasma membrane (PM) from activated (PM^{Act}) but not resting (PM^{rest}) T_h cells was found to induce B-cell RNA synthesis (Figure 1a); this induction, indicative of B-cell activation, was not affected by antibodies such as anti-LFA-1, anti-CD4, anti-ICAM-1. CD40-Ig or MR1, however, were found to be able to inhibit PM^{Act}-induced B-cell activation, as shown in Figure 1b and Figure 6.

The induction of B-cell activation may be measured by techniques such as [^{3}H]-uridine incorporation into RNA (as B-cells differentiate, RNA synthesis increases), or by [^{3}H]-thymidine incorporation, which measures DNA synthesis associated with cell proliferation. For optimal measurement of the effect of CD40CR on B-cell proliferation, interleukin-4 (IL-4) may be added to the culture medium at a concentration of about 10 ng/ml.

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Alternatively, B-cell activation may be measured as a function of immunoglobulin secretion. For example, CD40CR, in substantially purified form, or as present in PM, or otherwise, may be added to resting

5 B-cells together with IL-4 (10 ng/ml) and IL-5 (5 ng/ml). After three days of culture, an additional volume of culture medium may be added. On day 6 of culture, supernatant (SN) from individual cultures may be harvested and quantitated for IgM and IG₁ as

10 described in *Noelle et al., (1991) J. Immunol.* *146*:1118-1124.

5.2.3. CELLULAR DISTRIBUTION

CD40CR may also be characterized by its cellular

15 distribution. For example, CD40-Ig was observed to bind to activated, but not resting T_h1, as assessed by flow cytometry (Figure 3). Furthermore, CD40-Ig was observed to bind to Jurkat cells, HSB2 cells, and activated T-cells from human peripheral blood, but did

20 not appear to bind significantly to CEM cells, HPBALL cells, or murine thymoma cells.

For example, and not by way of limitation, the presence of CD40CR on a particular cell type ("test cells") may be evaluated by flow cytometry as follows.

25 Test cells may be tested in parallel with resting (negative control) and activated (positive control) T_h cells. All cells may be incubated at a concentration of about 1 x 10⁵ cells/50 μl with ligand (e.g. CD40-Ig or MR1) for 20 minutes at 4°C, followed by FITC-

30 conjugated anti-ligand antibody. Propidium iodide may be added to all samples to a final concentration of 2 μg/ml. Flow cytometric analysis may then be performed, for example on a BD FACSCAN. After positive gating of cells by forward versus side

35 scatter, and by red negativity (for propidium iodide

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exclusion), and the log green fluorescence of viable
cells may be ascertained.

5.3. PREPARATION OF PURIFIED CD40CR

5　　　The present invention provides for substantially
purified CD40CR. Such CD40CR may be prepared from
cells bearing CD40CR, such as activated helper T-
cells, Jurkat, and HSB2 cells, by the following method.

　　　Plasma membranes may be prepared from appropriate
10　cells, such as activated T_h1 cells, by discontinuous
sucrose gradient sedimentation, as described in *Noelle*
et al., 1991, *J. Immunol.* *146*:1118-1124. CD40CR may
then be isolated by dissociating the crude membrane
extract with mild detergent, and then performing size
15　exclusion chromatography followed by either affinity
chromatography using appropriate ligands (e.g. MR1 or
CD40-Ig) bound to a solid support, immunoprecipitation
(e.g. by CD40-Ig or MR1), and/or gel electrophoresis.

　　　The resulting protein may be expected to have a
20　molecular weight of about 39 kD.

　　　The present invention provides for a soluble
CD40CR (i.e. cell-free) which may be comprised in
pharmaceutical compositions together with a suitable
carrier. It further provides for CD40CR which is
25　linked to a second molecule which may be a peptide,
protein, lipid, carbohydrate, enzyme, toxin, growth
factor, lymphokine, antiproliferative agent,
alkylating agent, antimetabolite, antibiotic, vinca
alkaloid, platinum coordinated complex, radioisotope,
30　or a fluorescent compound.

　　　The present invention further provides for
substantially purified CD40CR which has been prepared
by chemical synthesis or recombinant DNA techniques.
For example, the gene for CD40CR may be isolated by
35　inserting cDNA prepared from activated helper T-cells

into the λgt10 expression system, and then screening with MR1 or CD40-Ig binding to identify CD40CR-expressing clones. Alternatively, cDNA prepared from activated helper T-cells may be transfected into COS cells, the supernatants of which may be screened with MR1 or CD40-Ig to identify CD40CR producers. The gene for CD40CR may be then used to express CD40CR using expression systems known in the art.

5.4. USES OF LIGANDS THAT BIND TO CD40CR

The present invention provides for methods of controlling B-cell activation that utilize ligands that bind to CD40CR. In particular, it provides for a method of inhibiting B-cell activation comprising exposing a mixture of B-cells and T_h cells to an effective concentration of ligand that binds to CD40CR. Ligands that may be used are described supra in section 5.1. The method of the invention may be practiced in vitro or in vivo. An effective concentration refers to a concentration of a ligand that inhibits B-cell activation, measured by any technique known in the art (including those set forth in section 5.2, supra) by at least about 30 percent, and preferably by about 75 percent. According to a preferred, specific, non-limiting embodiment of the invention, CD40-Ig may be used as ligand, in which case an effective concentration may be at least about 10 μg/ml. In another specific, nonlimiting embodiment of the invention, the monoclonal antibody MR1 may be used, in which case an effective concentration may be at least about 10 μg/ml. If the method is practiced in vivo, an effective concentration of ligand may refer to plasma concentration of ligand or to a local concentration. For example, it may be desirable to

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inhibit B-cell activation in a localized area in order
to limit the effects on the immune system as a whole.

In particular embodiments, the invention provides
for a method of treating a subject suffering from a
5   disorder associated with B-cell activation, comprising
administering to the subject a therapeutic amount of
ligand that binds to CD40CR. A subject may be a non-
human or, preferably, a human animal.

Disorders associated with B-cell activation
10   include, but are not limited to, allergy (including
anaphylaxis); autoimmune conditions including drug
induced lupus, systemic lupus erythematosus, adult
rheumatoid arthritis, juvenile rheumatoid arthritis,
scleroderma, Sjogren's Syndrome, etc.; and viral
15   diseases that involve B-cells, including Epstein-Barr
infection, and retroviral infection including
infection with a human immunodeficiency virus.

Because it has been suggested that B-cell
activation is associated with the induction of human
20   immunodeficiency virus replication from latency, it
may be desirable to administer the ligands of the
invention to HIV positive individuals who have not yet
developed AIDS or ARC.

Ligands may be administered, in a suitable
25   pharmaceutical carrier, by any method known in the
art, including intravenous, intraperitoneal,
subcutaneous, intrathecal, intraarticular or
intramuscular injection, and oral, intranasal,
intraocular and rectal administration, and may be
30   comprised in microspheres, liposomes, and/or sustained
release implants.

A therapeutic amount of ligand is defined as an
amount which significantly diminishes the deleterious
clinical effects of B-cell activation, and may vary
35   among ligands used and conditions treated. If CD40-Ig

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is used, therapeutic concentration may be about 10
μg/ml either systemically (plasma concentration) or
locally. If MR1 is used, a therapeutic concentration
may be about 10 μg/ml either systemically (plasma

5 concentration) or locally.

In a further embodiment of the invention, the
above methods may utilize a ligand comprising a toxin
or antimetabolite such that T_h cells are killed or
damaged and B-cell activation is decreased as a result

10 of T_h cell destruction.

The ligands of the invention may also be used to
label activated T cells, a technique which may be
useful in the diagnosis of T cell disorders. To this
end, ligand comprising an enzyme, radioisotope,

15 fluorescent compound or other detectable label may be
exposed to T cells in vitro or in vivo and the amount
of binding may be quantitated.

The ligands of the invention may also be used to
deliver substances, e.g. growth factors, to activated

20 T-cells.

5.5. USES OF CD40CR

The present invention provides for methods of
controlling B-cell activation that utilize CD40CR or a

25 molecule comprising CD40CR, prepared as described in
section 5.3, supra. In particular, it provides for a
method of promoting B-cell activation comprising
exposing B-cells to an effective concentration of
CD40CR. The method may be practiced in vivo or in

30 vitro. An effective concentration refers to a
concentration of receptor that induces B-cell
activation, measured by any technique known in the art
(including those set forth in section 5.3, supra) by

35 at least about 30 percent. In specific, nonlimiting

embodiments of the invention, the concentration of
CD40CR may be about 10 μg/ml locally or systemically.

In particular embodiments, the invention provides
for a method of treating a subject suffering from an

5 immunodeficiency disorder associated with diminished
humoral immunity, comprising administering to the
subject a therapeutic amount of CD40CR. A subject may
be a non-human or, preferably, a human animal.

Immunodeficiency disorders associated with

10 diminished humoral immunity include acquired
immunodeficiency syndrome, immunodeficiency associated
with malignancy or cachexia, iatrogenic
immunodeficiency caused, for example, by chemotherapy
or radiation therapy, as well as genetic disorders

15 involving humoral immunity.

CD40CR may be administered, in a suitable
pharmaceutical carrier, by any method known in the
art, including intravenous, intraperitoneal,
subcutaneous, intrathecal, intraarticular, or

20 intramuscular injection, and oral, intranasal,
intraocular, and rectal administration and may be
comprised in microspheres, liposomes, and/or sustained
release implants.

A therapeutic amount of CD40CR for CD40 is

25 defined as that amount which increases immunoglobulin
production by at least about 30 percent.

In a further embodiment, CD40CR may be conjugated
to a toxin, and then administered to a subject under
circumstances in which it would be preferable to

30 destroy B-cells that express CD40. Examples of such
circumstances include patients receiving organ
transplants or suffering from multiple myeloma or
another B-cell malignancy, or from autoimmune disease.

CD40CR may also be used to label B-cells

35 expressing CD40, a technique which may be useful in

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the diagnosis of B-cell disorders. To this end,
receptor linked to an enzyme, radioisotope,
fluorescent compound or other detectable label may be
exposed to B-cells _in vivo_ or _in vitro_ and the amount
of binding may be quantitated.

CD40CR may also be used to deliver molecules that
are linked to it to B-cells.

6.   EXAMPLE; A NOVEL RECEPTOR, CD40CR, ON ACTI-
     VATED HELPER T-CELLS BINDS CD40 AND TRANSDUCES
     THE SIGNAL FOR COGNATE ACTIVATION OF B-CELLS

6.1.   MATERIALS AND METHODS
6.1.1.   ANIMALS

Female DBA/2J mice (Jackson Laboratories, Bar
Harbor, ME) were used for the preparation of filler
cells to support the growth of T_h clones and in the
preparation of resting B-cells.

6.1.2.   HELPER T-CELL CLONES (T_h)

D1.6, a I-A^d-restricted, rabbit Ig-specific T_h1
clone (Kurt-Jones et al., (1987) J Exp Med 166:1774-
1787) was obtained from Dr. David Parker, University
of Mass. at Worcester. D1.6 will be referred to
herein as T_h1.

6.1.3.   ACTIVATION OF T_h BY ANTI-CD3

T_h1 were cultured (8 x10^6/well) in cluster wells
(6 well, Corning, NY) coated with 40 μg/4 ml of
PBS/well with anti-CD3 for 16 hours, as described in
(Noelle et al., (1991) J. Immunol. 146:1118-1124).

6.1.4.   PREPARATION OF T_h PLASMA MEMBRANES

Plasma membranes were prepared by discontinuous
sucrose gradient sedimentation, as described in
(Noelle et al., (1991) J. Immunol. 146:1118-1124).

6.1.5. PREPARATION OF RESTING B-CELLS

Resting splenic B-cells were prepared by
sedimentation on discontinuous Percoll gradients, as
described in (Defranco et al., (1982) J. Exp. Med.
5 155:1523). Cells isolated from the 70-75% (density of
1.087-1.097) Percoll interface were typically >95%
mIg⁺, had a uniform, low degree of near forward light
scatter and were unresponsive to Con A.

10 ### 6.1.6. ANTIBODIES

The following mabs were purified by ion exchange
HPLC from ascites fluid of mice which had been
irradiated and bone marrow reconstituted: anti-
CD3:145-2C11 (Leo et al., (1987) Proc. Natl. Acad.
15 Sci. USA 84:1374-1378); anti-α,β:H57-597; anti-CD4:
GK1.5 (Wilde et al., (1983) J. Immunol. 131:2178-
2183); anti-ICAM:YN1/1.7.4 (Prieto et al., (1989) Eur.
J. Immunol. 19:1551-1557); anti-LFA-1: FD441.8
(Sarmiento et al, (1982) Immunol. Rev. 68:135); and
20 anti-rat/hamster κ chain:RG-7 (Springer, (1982)
Hybrid. 1:257-273).

6.1.7. PREPARATION OF THE CD40 RECOMBINANT GLOBULIN (CD40-Ig)

25 The CD40 fusion protein was prepared by digesting
a plasmid containing a cDNA encoding the CD40 antigen
(Stamenkovic and Seed, (1989) EMBO J. 8:1403-1410)
with the restriction enzyme Pst I (P) and Sau 3A (S3).
This P/S3 fragment was subcloned into the same plasmid
30 digested with P and Bam H1 (B). This allowed the
preparation of the CD40Δ which encoded a CD40 protein
truncated upstream from the transmembrane domain. The
DNA fragment encoding a CD40Δ was then subcloned into
the immunoglobulin fusion plasmid (Aruffo et al.
35 (1990), Cell. 61:1303-1313) using a MluI and B digest.
The CD40-Ig fusion protein was produced by transient

transfection in COS cells and purified on a protein A
column as described in ((Aruffo et al., (1990) Cell.
61:1303-1313).

6.1.8. LYMPHOKINES

Interleukin 4 (IL4): Recombinant mouse IL4 was
generously provided by Drs. C. Maliszewski and K.
Grabstein, Immunex Corporation, Seattle, WA.

Interleukin 5 (IL5): Recombinant mouse IL5 was
purchased from R&D Research, Sarrento, CA.

6.1.9. INDUCTION OF B-CELL RNA SYNTHESIS BY ACTIVATED T_h PLASMA MEMBRANES

3×10^4 resting B-cells were cultured in 50 μl of
cRPMI in A/2 microtiter wells (Costar, Cambridge, MA).
To these wells, 0.5 μg of T_h1 or T_h2 membrane protein
was added. From 42-48 hrs, wells were pulsed with 2.5
μCi of ^{3}H-uridine (New England Nuclear, Boston
MA),harvested, and the radioactivity determined by
liquid scintillation spectroscopy. The results were
expressed as cpm/culture +/-s.d.

6.1.10. INDUCTION OF B-CELL IMMUNOGLOBULIN SECRETION BY ACTIVATED T_h PLASMA MEMBRANES AND LYMPHOKINES

Resting B-cells were cultured as described above.
To culture wells, 0.5 μg of T_h1 membrane protein, IL4
(10 ng/ml) and IL5 (5 ng/ml) were added. On day three
of culture, an additional 50 μl of cRPMI was added.
On day six of culture, SN from individual wells
were harvested and quantitated for IgM and IgG_1, as
described in (Noelle et al., (1991) J. Immunol.
146:1118-1124).

6.1.11. INDUCTION OF B-CELL PROLIFERATION BY
ACTIVATED T$_h$ AND IL4

4×10^4 resting B-cells were cultured in 50 μl of
cRPMI in A/2 microtiter wells (Costar, Cambridge, MA).
To these wells, 1×10^4 resting or activated, irradiated
(500 rads) T$_h$1 and IL4 (10 ng/ml) were added. On day
three of culture, wells were pulsed with 1 μCi of ^{3}H
thymidine, as described in (Noelle et al., (1991) J.
Immunol. 146:1118-1124).

6.1.12. PRODUCTION OF MONOCLONAL ANTIBODIES
SPECIFIC TO MEMBRANE PROTEINS
INDUCED ON ACTIVATED T$_h$1

Hamsters were immunized intraperitoneally with 5-
10 x 10^6 activated T$_h$1 (D1.6) at weekly intervals for
six weeks. When the serum titer against murine T$_h$1 was
greater than 1:10,000, cell fusions were performed
with polyethylene glycol using immune hamster
splenocytes and NS1. SN from wells containing growing
hybridomas were screened by flow cytometry on resting
and activated T$_h$1. One particular hybridoma, which
produced a mab that selectively recognized activated
T$_h$, was further tested and subcloned to derive MR1.
MR1 was produced in ascites and purified by ion
exchange HPLC.

6.1.13. FLOW CYTOFLUOROMETRIC ANALYSIS OF ACTIVATION
MOLECULES EXPRESSED ON T$_h$

Resting and activated T$_h$(16 hours with anti-CD3)
were harvested and incubated at 1×10^5 cells/50 μl with
fusion protein for 20 minutes at 4°C, followed by
FITC-conjugated goat anti-human (h)IgG (25 μg/ml,
Southern Biotechnology, Birmingham, AL). To all
samples, propidium iodide was added at final
concentration of 2 μg/ml. Flow cytofluorometric

analysis was performed on a BD FACSCAN. After
positive gating of cells by forward versus side
scatter, and by red negativity (for propidium iodide
exclusion), the log green fluorescence of viable cells
5   was ascertained. At least 5,000 viable cells were
analyzed for the determination of percent positive
cells and MFI. Staining with MR1 employed FITC-
conjugated RG7, a mouse anti-rat/hamster κ chain mab.

10   6.1.14.   BIOSYNTHETIC LABELLING, IMMUNOPRECIPITATION,
SDS-PAGE AND FLUOROGRAPHY

T_h1 were rested or activated with insolubilized
anti-CD3 for 16 hrs. Proteins from resting and
activated T_h (20 x 10^6/ml) were labelled with 1 mCi of
15   [^{35}S]-methionine/cysteine for one hour, at which time
they were washed twice in RPMI/10%FCS and the cell
pellet was lysed in extraction buffer, as described
(Noelle et al., (1986) J. Immunol. 137:1718-1726).
Purified antibodies or fusion proteins (1-10 μg) were
20   added to 500μl of lysate (5x10^6 cell equivalents) at
4°C for 16 hours. At that time, the lysates were
transferred to tubes containing 50 μl of packed
Protein A-sepharose. The pelleted Protein A-Sepharose
was resuspended and tubes were incubated at 4°C for 1
25   hr with agitation. The samples were then washed 3x
with high stringency wash buffer. The pelleted
protein A-Sepharose was resuspended in 30 μl of SDS
sample buffer and run on a 10% polyacrylamide gel.
After running the gel, the gel was fixed and
30   fluorography performed.

35

6.2. RESULTS

6.2.1. EFFECT OF MONOCLONAL ANTIBODIES ON THE INDUCTION OF B-CELL RNA SYNTHESIS BY PM^{Act}

In order to define the cell surface molecules that mediated the induction of B-cell cycle entry by PM^{Act}, mabs to T_h membrane proteins were added to cultures of PM^{Act} and B-cells. PM^{Act} induced B-cell RNA synthesis eight-fold over that observed with PM^{rest}) (Figure 1a). The addition of anti-LFA-1, anti-CD4, anti-ICAM-1, alone, or in combination, did not inhibit the induction of B-cell RNA synthesis by PM^{Act}.

6.2.2. CD40-Ig INHIBITED T_h-INDUCED B-CELL CYCLE ENTRY, DIFFERENTIA-TION AND PROLIFERATION

In the human system, it had been shown that anti-CD40 mab induced B-cell proliferation (Clark and Lane, (1991) Ann. Rev. Immunol. 9:97-127) thereby implicating CD40 as an important triggering molecule for B-cells. To determine if CD40 was involved in the induction of B-cell RNA synthesis by PM^{Act}, a soluble fusion protein of the extracellular domains of human CD40 and the F_c domain of human IgG_1 (CD40-Ig) was added to cultures of PM^{Act} and B-cells. PM^{Act} derived from T_h1 and T_h2 were prepared and used to stimulate B-cell RNA synthesis. The addition of CD40-Ig to culture caused a dose-dependent inhibition of B-cell RNA synthesis that was induced by PM^{Act} from T_h1 and T_h2 (Fig. 1b). Half-maximal inhibition of B-cell RNA synthesis induced by PM^{Act} from T_h1 and T_h2 was about 5 µg/ml CD40-Ig. A CD7E-Ig fusion protein (Damle and Aruffo, (1991) Proc. Natl. Acad. Sci. USA 88:6403-6407) was without effect even when used at 25 µg/ml.

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To investigate whether CD40-Ig inhibited the activation of B-cells by T-independent activators, B-cells were cultured in the presence of LPS and CD40-Ig. On day 2, RNA synthesis was assessed (Fig. 1c).

5   CD40-Ig was ineffective at inhibiting B-cell activation by LPS, yet inhibited the response of B-cells to PM^Act.

In the presence of PM^Act, IL4 and IL5, B-cells polyclonally differentiated to produce Ig (Hodgkin et

10   al., (1990) J. Immunol. 145:2025-2034; Noelle et al., (1991) J. Immunol. 146:1118-1124). To evaluate the requirements for CD40 signalling in this process, CD40-Ig was added at the initiation of culture, or on subsequent days of culture. The addition of CD40-Ig

15   (Fig. 2a) at the initiation of culture inhibited greater than 95% of polyclonal IgM and IgG₁ production compared to control levels in its absence. In contrast, the addition of CD40-Ig on day 1 and 2 of culture showed little, if any, inhibitory effect on

20   IgM and IgG₁ production. These data indicated that after 24 hours, signalling via CD40 is no longer essential for the differentiation of B-cells to Ig secretion.

Data thus far indicated that CD40 was implicated

25   in the activation of B-cells by PM^Act. Studies were performed in order to ensure that CD40 was also involved in the activation of B-cells by intact, viable, activated T₄. T₄1 were activated for 16 hours with insolubilized anti-CD3, harvested and irradiated.

30   The irradiated T₄1 were cultured with B-cells in the presence of IL4 and B-cell proliferation was determined on day 3 of culture. An exogenous source of IL4 was required to achieve B-cell proliferation with T₄1, because T₄1 do not produce IL4 (Noelle et

35   al., (1989) J. Immunol. 143:1807-1814). CD40-Ig

inhibited the induction of B-cell proliferation by irradiated T_h in a dose-dependent manner, similar to that observed with PM^{Aᶜ} (Fig. 2b). The negative control, CD7E-Ig, exerted no appreciable effect.

5

6.2.3. CD40-Ig DETECTED A MOLECULE EXPRESSED ON ACTIVATED, BUT NOT RESTING T_h

To investigate whether activated T_h1 express a binding protein for CD40, resting and activated (16 hours) T_h1 were stained with CD40-Ig or CD7E-Ig, followed by FITC-anti-HIgG. Binding of CD40-Ig was assessed by flow cytometry (Fig. 3). T_h1 that were activated for 16 hours with anti-CD3, but not resting T_h1, stained 56% positive with CD40-Ig, but not with the control CD7E-Ig. To identify the CD40-Ig binding protein, T_h1 proteins were biosynthetically labelled with [^{35}S]-methionine/cysteine and proteins immunoprecipitated with CD40-Ig or CD7E-Ig. The immunoprecipitated proteins were resolved by SDS-PAGE and fluorography (Figure 4). A prominent band with an apparent molecular weight of 39 kD immunoprecipitated in a dose-dependent manner with 1 and 10 µg of CD40/sample. As controls, anti-class I mab immunoprecipitated bands at 55 kD and a low molecular weight band, β2 microglobulin. In the absence of mab, no prominent bands were visible. A 39 kd band was also immunoprecipitated from activated T_h that were vectorially labelled with ^{125}I, confirming that the 39kD protein was a membrane protein.

6.2.4. MONOCLONAL ANTIBODY MR1, SPECIFIC TO 39Kd T_h MEMBRANE PROTEIN, INHIBITED THE INDUCTION OF B-CELL RNA SYNTHESIS BY PM^{Aᶜ}

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Mabs specific to antigens selectively expressed on activated versus resting T_h were developed to

identify T_h molecule(s) responsible for the T_h effector
phase activity. One such mab, MR1, recognized an
antigen that was selectively expressed on activated
T_h1. To investigate whether MR1 and CD40-Ig recognized
the same molecule, flow cytometry and blocking studies
were performed. CD40-Ig and MR1 stained approximately
56% and 61%, respectively, of activated, but not
resting Th (Fig. 5a). MR1, but not another hamster
anti-T cell mab, anti-α/β TCR, blocked the staining of
activated T_h1 with CD40-Ig, in a dose-dependent manner.
These data suggested that CD40-Ig and MR1 recognized
overlapping or identical epitopes on the 39 kD Th
protein. To further demonstrate that CD40-Ig and MR1
recognized the same molecule, the antigen that bound
MR1 was identified by immunoprecipitation of proteins
from radiolabelled Th lysates. Both CD40-Ig and MR1
immunoprecipitated a 39 kD protein (Fig. 5b).
Finally, immunoprecipitation of the 39kD protein with
CD40-Ig removed the antigen recognized by MR1 from
radiolabelled lysates of activated T_h supporting the
tenet that the MR1 antigen and the CD40 binding
protein were identical.

Functional studies were performed with MR1 to
address whether this mab neutralized the activity
expressed by PM^{Ad}. PM^{Ad} and B-cells were cultured
alone, or in the presence of hamster mabs or CD40-Ig.
Two hamster mabs, anti-α/β TCR and α-CD3 did not
inhibit the activation of resting B-cells by PM^{Ad}. In
contrast, MR1 or CD40-Ig inhibited B-cell activation
(Fig. 6).

6.3.   DISCUSSION
The data show that blocking of prominent T_h
surface molecules (LFA-1, CD4, ICAM-1, CD3, α,β TCR)
with mabs did not impede the capacity of activated T_h

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to induce B-cell cycle entry. In contrast, CD40-Ig or a maB specific to the CD40 binding protein, blocked T_h-dependent B-cell activation in a dose-dependent manner. Furthermore, the CD40 binding protein was

5 identified as a 39 kD protein that is selectively expressed on the membranes of activated, but not resting T_h. Both CD40-Ig and a mab specific to the 39kD CD40 binding protein blocked B-cell activation by PM^{Act}.

10 Although a number of membrane proteins have been implicated in T_h-dependent B-cell signalling, evidence presented herein dismisses the contribution of some molecules (LFA-1, CD4, CD3, α,β TCR, ICAM-1) and implicates CD40 as the B-cell receptor for cognate

15 signalling by T_h. Data show that CD40-Ig and a mab specific to the CD40 binding protein inhibits T_h-dependent B-cell activation.

The ligand for CD40 is a 39Kd protein that is expressed on activated, but not resting T_h.

20 Biochemical studies indicate that the 39kD protein is a single chain molecule since electrophoretic migration was not influenced by reducing agents. Based on the functional studies presented in this study, both activated T_h1 and T_h2 express the 39 kD

25 CD40 binding protein. This is consistent with the functional studies that show both T_h1 and T_h2 induce B-cell cycle entry. In an attempt to further characterize the 39 kD protein, cDNA encoding CD proteins in the MW range of 39kD (CD 53, CD37 and

30 CD69) were transiently transfected into COS cells and the cells were tested for CD40-Ig binding. None of the transfected COS cells expressed proteins that bound CD40-Ig. It is therefore suspected that the 39 kD protein is not one of these CD proteins.

35

The biochemical basis for signal transduction between T_h and B-cells has been elusive. The identification of CD40 as the signal transducing molecule for T cell help focusses attention on

5　specific biochemical pathways known to be coupled to the CD40 molecule. CD40 is a member of the nerve growth factor receptor (NGFR) family by virtue of the presence of four cysteine-rich motifs in its extracellular region. Signaling through CD40 by mab

10　has been shown (Uckun et al., (1991) J. Biol. Chem. 266:17478-17485) to involve the activation of tyrosine kinases resulting in the increased production of inositol trisphosphate and the activation of at least four distinct serine/threonine kinases. Based on

15　information obtained from signaling through other members of the NGF receptor family, it is anticipated that interaction between activated T_h and B will result in many of the same biochemical processes.

20　7. EXAMPLE: BINDING OF CD40 Ig TO HUMAN T-CELL LINES

For immunofluorescence binding studies, CD40 Ig fusion protein was conjugated with biotin using biotin-succinimide (Sigma). Flow cytometry analysis was then performed by tow-step staining using

25　phycoerythrin (PE)-strepavidin (Bectin-Dickinson) with a Coulter Epics C instrument. Representative results of screening multiple T cell lines is presented below. The Jurkat and HSB2 cell lines were found to bind specifically, whereas other T cell lines including

30　CEM, HPBALL, and murine thymoma did not bind the CD40 Ig fusion protein (Fig. 7).

Various publications are cited herein which are hereby incorporated by reference in their entirety.

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